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DTIC FILE

Form Approved
OMB No 0704-0188
Exp. Date Jun 30, 1986

(1)

AD-A223 968

1a. REPORT
UNCL

2a. SECURITY

2b. DECLASSIFICATION / DOWNGRADING SCHEDULE

JUL 18 1990

D

4. PERFORMING ORGANIZATION REPORT NUMBER

6a. NAME OF PERFORMING ORGANIZATION
Dept Hazardous Microorganisms
Div Comm Dis & Immunology, WRAIR

6b. OFFICE SYMBOL
(if applicable)
SGRD-UWF-I

6c. ADDRESS (City, State, and ZIP Code)
Washington, DC 20307-5100

8a. NAME OF FUNDING / SPONSORING
ORGANIZATION
WRAIR

8b. OFFICE SYMBOL
(if applicable)

8c. ADDRESS (City, State, and ZIP Code)

5. MONITORING ORGANIZATION REPORT NUMBER(S)

7a. NAME OF MONITORING ORGANIZATION

Walter Reed Army Institute of Research
Walter Reed Army Medical Center

7b. ADDRESS (City, State, and ZIP Code)
Washington, DC 20307-5100

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
63763A	3M263763D810	AI	131

11. TITLE (Include Security Classification) Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection (U)

12. PERSONAL AUTHOR(S) B.M. Kaufman, P.L. Summers, D.R. Dubois, W. Houston Cohen, M.K. Gentry, R.L. Timchak, D.S. Burke, and K.H. Eckels

13a. TYPE OF REPORT	13b. TIME COVERED FROM _____ TO _____	14. DATE OF REPORT (Year, Month, Day) 1989 September 15	15. PAGE COUNT
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16. SUPPLEMENTARY NOTATION

17. COSATI CODES

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)

FIELD	GROUP	SUB-GROUP

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Five murine monoclonal antibodies (Mabs) reactive against the prM glyco-proteins of DEN-3 and -4 were used to passively protect mice in vivo against lethal challenge with homologous and heterologous dengue virus serotypes. Four of the 5 prM-reactive monoclonals cross-protected mice against heterologous challenge, whereas 1 protected against challenge with only the homologous serotype. Although in vitro binding to virions was readily demonstrated, only 2 of the prM Mabs had detectable neutralizing activity. The neutralizing activity could not be enhanced by anti-mouse immunoglobulin or complement. However, 4 of the 5 prM Mabs fixed complement. This is the first report of prM-specific Mabs that are protective in mice.

JFS

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT

UNCLASSIFIED/UNLIMITED SAME AS RPT. DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION

UNCLASSIFIED

22a. NAME OF RESPONSIBLE INDIVIDUAL

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22b. TELEPHONE (Include Area Code)

301/427-5208

22c. OFFICE SYMBOL

SGRD-UWF-I

MONOCLONAL ANTIBODIES FOR DENGUE VIRUS prM GLYCOPROTEIN PROTECT MICE AGAINST LETHAL DENGUE INFECTION

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Abstract. Five murine monoclonal antibodies (Mabs) reactive against the prM glycoproteins of DEN-3 and -4 were used to passively protect mice *in vivo* against lethal challenge with homologous and heterologous dengue virus serotypes. Four of the 5 prM-reactive monoclonals cross-protected mice against heterologous challenge, whereas 1 protected against challenge with only the homologous serotype. Although *in vitro* binding to virions was readily demonstrated, only 2 of the prM Mabs had detectable neutralizing activity. The neutralizing activity could not be enhanced by anti-mouse immunoglobulin or complement. However, 4 of the 5 prM Mabs fixed complement. This is the first report of prM-specific Mabs that are protective in mice.

Dengue virus is a mosquito-borne flavivirus of great medical importance due to its wide global distribution and the frequency of DEN epidemics.¹ Several experimental live-attenuated vaccines have been tested in humans and appear to be safe and immunogenic.^{2,3} Since there are 4 serotypes of dengue, each serotype must be represented in a vaccine to confer complete protection. Future efficacy trials will determine whether these vaccines will protect against natural infection.

In our efforts to characterize dengue epitopes that are important for immune response and protection, we have used monoclonal antibodies (Mabs) directed at immunogenic viral proteins. Using passive protection assays in mice, Mabs specific for the DEN-2 envelope (E) glycoprotein were found to be protective.⁴ These Mabs had a spectrum of cross-reactivity against other DEN serotypes. In the current studies, we found that another structural protein, prM, also appears to contain protective epitopes. A panel of prM-specific Mabs for DEN-3 and DEN-4 viruses was used to assess homologous and heterologous protection and to characterize the Mabs in various antigen binding and functional assays of reactivity.

MATERIALS AND METHODS

Cell cultures and virus

Aedes albopictus clone C6/36 cells were maintained at 28°C in 32 oz glass prescription bottles

in Eagle's minimum essential medium (GIBCO, Grand Island, NY) containing 2% fetal bovine serum (M. A. Bioproducts, Walkersville, MD) and supplemented with 100 U penicillin and 100 µg streptomycin per ml. We used virus strains DEN-1 (Western Pacific 1974), DEN-2 (PR-159), DEN-3 (H87), and DEN-4 (341750). Cells were infected at multiplicities of 0.1-1.0 and virus harvested at 5-7 day intervals starting on day 5 postinfection.

Viruses were purified by sucrose density-gradient centrifugation and cell lysates prepared as previously described.⁴

Monoclonal antibodies

The production and partial purification of the anti-DEN Mabs have been described.⁵

Electrophoresis and immunoblotting

Purified virion preparations and cell lysates were separated on 12% polyacrylamide gels as previously described.⁶ The separated proteins were blotted onto nitrocellulose,⁷ probed with a 1:100 dilution of Mab, and visualized immunoenzymatically.⁸

Determination of antigen binding activity

Antigen binding activity of the Mabs was determined by an antibody-capture solid phase immunoassay. Polystyrene microtiter plates were

sensitized with goat antisera to mouse IgG diluted in carbonate buffer. After washing with 0.05% Tween-20 in phosphate-buffered saline (PBS), the plates were blocked with 1% BSA in PBS. Test antibody (cell culture supernatant or ascites fluid) was added for 2 hr, the plate was washed, and test antigen (derived from supernatants of dengue infected mosquito cells) in PBS plus 10% acetone-extracted human serum was added overnight. After washing, the plates received ^{125}I - or peroxidase-labeled human flavivirus hyperimmune IgG for 2 hr. Normal mouse ascitic fluid was used as a negative control; the broadly flavivirus-reactive Mab 4G2 was used as the positive control.

ELISA, neutralization assay, and complement fixation assay

The solid-phase enzyme-linked immunosorbent assay (ELISA) used for titration of the Mabs has been described.⁴

Virus neutralization (N) titers of the Mabs were determined by a plaque reduction neutralization test (PRNT) as described,⁹ using a 0.5 hr incubation time at 35°C.

Complement fixation (CF) titers of the Mabs were determined using a microtiter modification of the procedure of Kent and Fife.¹⁰

Mouse protection assay

The in vivo protective ability of the Mabs against DEN-1, -2, and -4 was assayed in BALB/cJ mice (Jackson Laboratories, Bar Harbor, ME) as previously described.^{4,11} Protection against DEN-3 challenge was assayed in suckling mice: 4-day-old animals were injected ip with 0.05 ml ascites fluid and challenged on the following day with $>100 \text{ LD}_{50}$ DEN-3 virus.

RESULTS

Viral protein specificity of Mabs

The viral protein specificities of the anti-DEN Mabs were determined by immunoblotting electrophoretically-separated proteins from virions and dengue infected cell lysates. Five Mabs, 14E9, 4H9, 2H2, 5C9, and 15H5, reacted with an 18–20 kDa protein present in both virions and cell lysates, which corresponds to prM protein. A representative immunoblot, using Mab 14E9, is

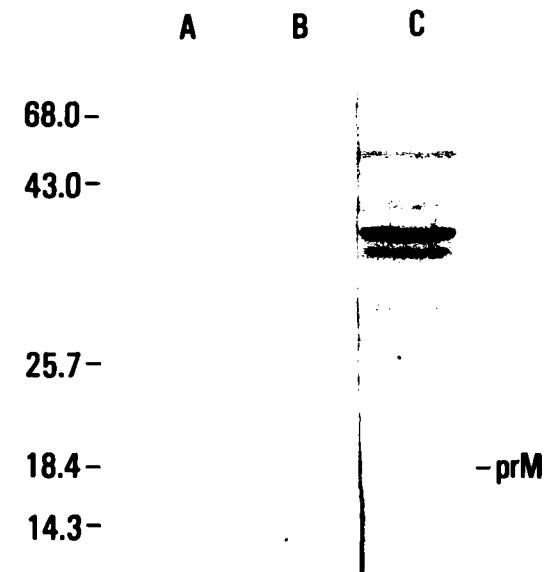


FIGURE 1. Western blot analysis of a DEN-3 infected cell lysate with prM-specific Mab 14E9. DEN-3 infected *Aedes albopictus* clone C6/36 cells were lysed, and the clarified lysate was subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were blotted to nitrocellulose, probed with Mab 14E9 and hyperimmune mouse ascitic fluid (HMAF), and developed as described in the text. Lane A contains molecular weight standards (shown in kilodaltons). Lane B, Mab 14E9. Lane C, DEN-3 HMAF. The position of the prM protein is indicated.

shown in Figure 1. The reactivity spectrum of each Mab with each dengue virus serotype was determined using antigen capture RIA or ELISA. Mabs 15H5, 14E9, 2H2, and 5C9 reacted with all members of the DEN complex; Mab 4H9 reacted with only DEN-1 and DEN-4 (Table 1).

In vitro activities of monoclonal antibodies

Generally, all the Mabs possessed titers of $\geq 10^5$ when assayed by ELISA against homologous purified virions (Table 1). Although the reactivity of the Mabs with virions was quite high, only 2 Mabs, 2H2 and 5C9, had any neutralizing activity. This low level of neutralizing activity was repeatable in several neutralization assays, but could not be enhanced by the addition of either anti-mouse immunoglobulin or guinea pig or mouse complement (data not shown). However, 4 of the 5 Mabs were able to fix complement in standard CF tests (Table 1).

TABLE 1

In vitro immunological characteristics of prM monoclonal antibodies

Mab	Antigen binding spectra*	Immunizing virus serotype	Neutralizing titer	Virion binding	CF titer
15H5	C	DEN-3	<10	10 ⁶	8
14E9	C	DEN-3	<10	10 ⁵	<8
2H2	C	DEN-4	10	10 ⁶	128
5C9	C	DEN-4	10	10 ⁶	8
4H9	SC	DEN-4	<10	10 ⁷	8

* C = Dengue complex-reactive; SC = dengue subcomplex-reactive.

Mouse protection assays

Cross-protection by the prM Mabs was assayed by challenging passively immunized mice with both homologous and heterologous virus (Table 2). In all cases, Mabs specific for a given serotype of dengue were able to protect against homologous challenge. Different patterns of cross-protection were demonstrated by the Mabs: Mab 4H9 was serotype specific, 14E9 and 5C9 were sub-complex protective, and 2H2 and 15H5 were complex protective (Table 2). Thus, there appear to be at least 3 epitopes on prM which differ in their protective capacity. These in vivo data appear to conflict with the in vitro antigen binding data which indicate that 4 of the 5 Mabs are complex-reactive (Table 1).

DISCUSSION

We and others have previously reported that murine Mabs directed against DEN-2 E-glycoproteins could passively protect mice against lethal DEN-2 challenge.^{4,12} Additionally, passive protection has been demonstrated in mice by Mabs against E-glycoprotein of Saint Louis encephalitis virus,¹³ and yellow fever virus.^{12,14} Mabs against the 48 K non-structural protein of yellow fever virus and DEN-2 have also been shown to protect mice against lethal challenge.¹⁴ In this study, we show that Mabs against the prM glycoprotein of dengue virus can also be protective against challenge with both homologous and heterologous virus. There was a good correlation between in vitro neutralizing activity and passive protection using E-specific Mabs for DEN-2;⁷ however, we were able to demonstrate significant passive protection in the current studies using Mabs that displayed little or no in vitro neutralizing activity (Tables 1, 2). Others have

TABLE 2

*Protection of mice by passive administration of monoclonal antibodies**

Mab	DEN-1 (No. survivors/no. challenged)	DEN-2 (No. survivors/no. challenged)	DEN-3 (No. survivors/no. challenged)	DEN-4 (No. survivors/no. challenged)
15H5	5/5	4/5	7/7	4/5
14E9	1/5	0/5	7/7	10/10
2H2	5/5	5/5	6/9	10/10
5C9	4/5	5/5	1/8	13/13
4H9	0/5	0/5	0/6	10/10
Normal ascites fluid	0/10	0/10	1/8	0/10

Mice were given Mabs followed by intracerebral challenge with DEN virus 1 day later.

reported the failure of neutralizing antibodies to protect against virus challenge,¹⁷ and the relationship between neutralizing activity and protection remains unclear.

Aaskov and others have recently reported that passive administration of Mab 2H2 failed to protect mice against DEN-1 challenge.¹⁸ Even though circulating reactive antibody could be demonstrated, the animals were given only antibody-containing supernatant fluids from the hybridoma cell line, and the concentration of active antibody may have been too low to elicit protection. Also, Mab 2H2 has been shown to lead to enhancement of infection under some circumstances.¹⁹ Thus, it is possible that a low dose of passively-administered antibody could be non-protective, or lead to infection enhancement, whereas a higher dose would result in protection as seen in our challenge studies.

The mechanism by which anti-prM Mabs protect against virus challenge is unknown. The general mechanism of virus neutralization by E-glycoprotein-directed Mabs has not been elucidated.²⁰⁻²³ Likewise, little is understood about protection mediated by other means. Hollins and Porterfield²⁴ have described the ability of polyclonal antisera to interfere with a fusion event between the membrane of endocytosed West Nile virus and a prelysozomal endosome required for viral replication. PrM Mabs 2H2 and 5C9 were not able to prevent DEN virus-induced cell fusion.²⁵ Therefore, the mechanism of fusion, however important this event is to the virus entry process, is not interfered with by dengue prM Mabs. One other possible mechanism of protection may involve the binding of antibodies to prM protein in the infected cell membranes, leading to activation of complement. Four

of the 5 Mabs used in the present study had CF activity (Table 1). Complement activation could then lead to lysis of the virus infected cell by antibody-dependent cytolysis and the abrogation of infection. NS1-specific Mabs for yellow fever 17D virus have been shown to be protective for mice.¹⁵ These Mabs, when augmented by the addition of complement, are cytolytic for yellow fever virus-infected target cells. However, 2 of the prM Mabs described (2H2 and 5C9) were not cytolytic in the same type of assay (J. Schlesinger, University of Rochester, NY, personal communication). The role of complement, if any, in the proposed mechanism might be elucidated using decomplemented (i.e., cobra venom factor-treated) mice.

Active immunization against DEN-2 non-structural protein NS1 conferred protection in mice against lethal DEN-2 challenge,¹⁶ and immunization of monkeys with yellow fever virus NS1 led to protection against yellow fever-induced hepatitis upon challenge with yellow fever virus.²⁵ However, the protection conferred by the DEN-2 NS1 was not cross-protective: mice challenged with the heterologous DEN-1 virus were not spared.¹⁶ The cross-protective nature of the Mabs against prM (Table 2) indicates that cross-reactive epitopes are present on the prM protein. Active immunization of mice with electrophoretically-purified DEN-1 prM failed to confer protective immunity.¹⁸ However, we and others²⁶ have found that use of detergent (SDS)-containing proteins as immunogens often results in a lack of protective immunity, even though antibodies can be demonstrated in the immunized animal's serum. We are purifying DEN-2 prM protein, using detergent-removal and other techniques to preserve immunogenicity, to determine whether active immunization with this protein can protect against virus challenge.

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Justification _____	
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Availability Codes	
Dist	Avail and / or Special
A-1	20

